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Effect of cloned inhibitor-resistant TEM β-lactamases on the susceptibility of *Haemophilus influenzae* to amoxicillin/clavulanate

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***Objectives*: To determine the effect of cloned inhibitor-resistant TEM β-lactamases (IRTs) on the susceptibility of *Haemophilus influenzae* to amoxicillin/clavulanate.**

***Methods*: IRT-2, -4 and -5 genes with various promoters were cloned into control strains of *H. influenzae* and the amoxicillin/clavulanate MICs were measured using Etests.**

***Results*: IRT enzymes were able to raise the amoxicillin/clavulanate MICs to between 0.38/0.19 and 4.0/2.0 mg/L depending on the IRT and promoter genotype, compared with MICs of 0.19/0.09 to 0.5/0.25 mg/L for the corresponding strains with TEM-1. Strains with an IRT and altered penicillin-binding proteins had amoxicillin/clavulanate MICs as high as 8.0/4.0 mg/L.**

***Conclusions*: Cloned IRT enzymes in *H. influenzae* raise the amoxicillin/clavulanate MICs to an extent comparable to naturally occurring strains with decreased amoxicillin/clavulanate susceptibility.**

Keywords: *H. influenzae*, IRTs, susceptibility

Introduction

The most common mechanism of ampicillin resistance in *Haemophilus influenzae* is via production of TEM-1 β-lactamase with a recent global survey reporting a prevalence of ~17%.[1] One strategy used to overcome this resistance is to use amoxicillin in combination with the β-lactamase inhibitor clavulanate, and this combination is widely prescribed to treat a range of respiratory tract infections caused by β-lactamase-producing strains of *H. influenzae*.[2] Despite the prevalence of TEM-1 β-lactamase in *H. influenzae* and the widespread use of amoxicillin/clavulanate, it has been surprising that inhibitor-resistant TEM β-lactamases (IRTs) and associated amoxicillin/clavulanate resistance has not been reported in *H. influenzae* as it has in Enterobacteriaceae.[3,4] This is especially so given that amoxicillin/clavulanate resistance has emerged in β-lactamase-negative ampicillin-resistant (BLNAR) and β-lactamase-positive amoxicillin/clavulanate-resistant (BLPACR) strains of *H. influenzae* as a result of altered penicillin-binding proteins (PBPs).[5,6] It has been suggested that the failure of IRTs to emerge in *H. influenzae* might be associated with the high intrinsic activity of penicillins against the organism and relatively small amounts of β-lactamase usually produced,[3,4] such that a level of resistance sufficient to allow for selection of mutants during therapy might not be produced.

In order to determine the effect that IRTs might have on the susceptibility to ampicillin and amoxicillin/clavulanate, a range of IRTs were artificially introduced into control strains of *H. influenzae*.

Methods

Construction of bla_{TEM-1} and bla_{IRT} library

Strains of TEM-1 β-lactamase-positive *H. influenzae*, previously characterized with regard to promoter genotype,[7,8] were used as PCR templates to clone bla_{TEM-1} with Pa/Pb, Pdel or Prpt promoters onto the shuttle vector pLS88 and into *Escherichia coli* XL1-Blue cells (Stratagene) as previously described.[9]

Plasmids were extracted from these clones and used as template for site-directed mutagenesis using the QuikChange Site Directed Mutagenesis Kit (Stratagene) and primers listed in Table 1 to generate bla_{IRT-2} and bla_{IRT-5} coding for IRT-2 (TEM-30) and IRT-5 (TEM-33) in *E. coli* XL1-Blue cells. Plasmid containing bla_{IRT-5} was extracted and subjected to another round of site-directed mutagenesis to generate bla_{IRT-4} coding for IRT-4 (TEM-35).

Finally, all constructed plasmids (pLS88 with bla_{TEM-1} and $bla_{IRT-2,\ -4}$ and $_{-5}$) were used to transform *H. influenzae* Rd. Plasmids containing bla_{TEM-1}, bla_{IRT-4} and bla_{IRT-5} in association with the Pdel promoter were also used to transform *H. influenzae* Rd that had previously been transformed with *ftsI* gene PCR products from a BLNAR strain. All transformations were performed by

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Table 1. Details of site-directed mutagenesis

Primers[a]	Mutation introduced[b]	Amino acid change[c]	IRT type
GGTGAGCGTGGGTCT**A**GCGGTATCATTGCAGCACTGG	C929**A**	Arg-244→Ser	IRT-2
CCCGAAGAACGTTTTCCAATG**C**TGAGCACTTTTAAAGTTC	A407**C**	Met-69→Leu	IRT-5
GGCAACTATGGATGAACGA**G**ATAGACAGATCGCTGAG	A1022**G**	Asn-276→Asp	IRT-4[d]

[a]Primers are listed 5′ to 3′ with mutation nucleotide in bold and underlined.
[b]Nucleotides are numbered according to Sutcliffe.
[c]Amino acids are numbered according to Ambler.
[d]IRT-4 mutagenesis was performed on bla_{IRT-5} to give the dual mutations A407C and A1022G in bla_{IRT-4}.

electroporation as described by Ubukata *et al*.,[5] and transformants were selected on chocolate agar supplemented with 30 mg/L kanamycin.

Validation of constructs

The presence of the respective bla_{TEM-1} and bla_{IRT} genes in the *E. coli* strains was confirmed by appropriate MICs of ampicillin and amoxicillin/clavulanate and by *bla* gene sequencing. β-Lactamase production in the *H. influenzae* transformants was confirmed by nitrocefin hydrolysis (Oxoid, Australia).

Susceptibility tests

MICs of ampicillin, amoxicillin/clavulanate and cefotaxime (for *Haemophilus* only) were determined using Etest strips according to the manufacturer's instructions (Australian Laboratory Services, Melbourne). *H. influenzae* ATCC 49247 and ATCC 49766 were used as quality control strains.

Results and discussion

The enzymes IRT-2, -4 and -5 were chosen for this study because they are representative of enzymes with substitutions at positions 69, 244 and 276 identified by computerized modelling as positions that are associated with inhibitor resistance via different molecular interactions.[3] Substitutions at these positions are also those most frequently reported in clinically derived IRTs.[10] Promoters Pa/Pb, Pdel and Prpt were chosen because they have been identified as the most common promoters in bla_{TEM-1} genes in *H. influenzae*.[7,8]

Table 2. MICs (mg/L) for *H. influenzae* with various *bla* genes

Host strain	*bla* gene	Promoter	Ampicillin	Amoxicillin/clavulanate
Rd	TEM-1	Pa/Pb	16	0.19/0.09
		Pdel	128	0.5/0.25
		Prpt	64	0.38/0.19
	IRT-2	Pa/Pb	2	0.38/0.19
		Pdel	32	2.0/1.0
		Prpt	32	2.0/1.0
	IRT-4	Pa/Pb	2	0.5/0.25
		Pdel	128	1.5/0.75
		Prpt	64	1.0/0.5
	IRT-5	Pa/Pb	16	1.5/0.75
		Pdel	128	4.0/2.0
		Prpt	128	4.0/2.0
Rd BLNAR	TEM-1	Pdel	128	2.0/1.0
	IRT-4	Pdel	256	8.0/4.0
	IRT-5	Pdel	256	8.0/4.0

In *E. coli* XL-1, the ampicillin MICs for the strains expressing any of the cloned bla_{TEM-1} and $bla_{IRT-2,\ -4}$ and $_{-5}$ constructs were >256 mg/L, and the amoxicillin/clavulanate (2:1 ratio) MICs ranged from 8/4 to 32/16 mg/L for strains expressing bla_{TEM-1} and 24/12 to 128/64 mg/L for $bla_{IRT-2,\ -4}$ and $_{-5}$. These MIC data are consistent with similar naturally occurring strains,[4,11] and, in conjunction with sequence data confirming the presence of appropriate promoters and IRT-related substitutions, validate the constructs as representative of naturally occurring resistance genes.

In *H. influenzae*, the amoxicillin/clavulanate MICs for strains expressing various cloned bla_{IRTs} were between 2- and 10-fold higher than for strains expressing bla_{TEM-1} from the same promoters (Table 2). All the strains expressing cloned bla_{IRTs} had amoxicillin/clavulanate MICs between 2- and 5-fold higher when associated with either the strong Pdel or Prpt promoters compared with the relatively weaker Pa/Pb promoters. This is consistent with what is seen in naturally occurring IRT-producing strains of *E. coli*, where the relatively stronger Pa/Pb or P4 promoters occur more frequently than the weaker P3 promoter.[12] Stronger promoters are probably selected for because of the decreased catalytic efficiency reported for many IRT enzymes,[4] where additional enzyme production is not only necessary to produce significant amoxicillin/clavulanate resistance but also to compensate for a relative loss of activity against ampicillin. In the *H. influenzae* strains in this study, this loss of catalytic efficiency is manifested as lower ampicillin MICs for IRT-2- compared with TEM-1-producing strains, which is consistent with the lower catalytic efficiency of IRT-2 compared with IRT-4 and IRT-5 and TEM-1.[4] Such decreases are not observed in the *E. coli* constructs in this study, or in naturally occurring IRT-positive *E. coli* strains because the ampicillin MICs are usually above the highest concentrations measured.

The cefotaxime MICs for all *H. influenzae* clones were between 0.008 and 0.016 mg/L with no evidence of decreased susceptibility associated with production of IRT-2, -4 or -5 enzymes compared with TEM-1.

When IRT enzymes were expressed in BLNAR strains of *H. influenzae*, the amoxicillin/clavulanate MICs were 2–5-fold higher than those for otherwise identical non-BLNAR strains.

A similar finding was observed when TEM-type extended-spectrum β-lactamases (ESBLs) were cloned into *H. influenzae*, with significantly higher MICs of cefotaxime observed when the ESBL was expressed in a BLNAR background.[13] Significantly, the only report of a naturally occurring ESBL in *Haemophilus* has been in two strains of *Haemophilus parainfluenzae* with TEM-16 and altered PBPs similar to those found in BLNAR strains of *H. influenzae*,[14] so a background of altered PBPs in BLNAR/BLPACR strains of *H. influenzae* might favour the emergence of IRT enzymes.

It is difficult to gauge the significance of the decreased susceptibility to amoxicillin/clavulanate of the IRT-producing *H. influenzae* strains in this study because the MICs cluster around the breakpoints for amoxicillin/clavulanate used by various regulatory bodies. All the strains are susceptible according to CLSI breakpoints ($S \leq 4$, $R \geq 8$ mg/L) but some would be considered resistant according to either the BSAC breakpoints ($S \leq 1$, $R > 1$ mg/L) or the recently proposed PK/PD breakpoints ($S \leq 2$, $R \geq 4$ mg/L).[2,15,16] The significance of the decreased susceptibility might best be considered in the context of comparisons to other naturally occurring strains. The strains produced in this study had amoxicillin/clavulanate MICs ranging from 0.38/0.19 to 4.0/2.0 mg/L, whereas the amoxicillin/clavulanate MIC_{90} for 8500 strains from the Alexander study 1998–2000 was 1.0/0.5 mg/L,[17] and the MIC_{90} was 2.0/1.0 for 2000 β-lactamase-positive strains surveyed by Farrell *et al*. in 2000–03.[18] In addition, the amoxicillin/clavulanate MICs for 108 BLNAR and BLPACR strains characterized by Dabernat *et al*.[6] ranged from 0.12/0.06 to 4.0/2.0 mg/L. On this basis it can be concluded that IRT enzymes in *H. influenzae* could produce resistance to amoxicillin/clavulanate significantly greater than baseline susceptible strains, and equivalent to that shown by currently existing BLNAR and BLPACR strains.

If IRTs in *H. influenzae* can produce a similar decrease in amoxicillin/clavulanate susceptibility to that seen in naturally occurring BLPACR and BLNAR strains, then why have IRTs not been detected? One possible reason is that the decreased amoxicillin/clavulanate susceptibility in BLNAR and BLPACR strains is not actually an evolutionary response to selective pressure of amoxicillin/clavulanate use, but rather an incidental finding associated with the altered PBPs and decreased cephalosporin susceptibility selected for by widespread cephalosporin use. This is consistent with the observation that strains with altered PBPs are more common in Japan where oral cephalosporin use is favoured over amoxicillin and amoxicillin/clavulanate compared with the United States and Europe.[2]

It is also possible that IRT-mediated decreased amoxicillin/clavulanate susceptibility has emerged and gone undetected, particularly given that the MICs will not exceed the current CLSI resistance breakpoint.

A lack of consensus on breakpoints and incomplete correlation between phenotypic and genotypic characterization has created difficulties and inconsistencies in detecting currently existing BLNAR and BLPACR strains.[2] In this context, detecting the possible emergence of IRT-producing strains might also be difficult. A clinical isolate that is β-lactamase-positive with reduced amoxicillin/clavulanate susceptibility or resistance is most probably a BLPACR strain, but could be a strain producing an IRT. The major differentiating characteristic would be that as a result of the altered PBP3, BLPACR strains would usually also show reduced susceptibility or resistance to cephalosporins whereas IRT-producing strains in this study did not. Confirmation of IRT production would require sequencing of the bla_{TEM} gene.

In conclusion, IRT enzymes in *H. influenzae* could cause a significant decrease in amoxicillin/clavulanate susceptibility but would be difficult to detect using current CLSI breakpoints. Laboratories should consider the presence of an IRT in β-lactamase-positive strains of *H. influenzae* with decreased susceptibility to amoxicillin/clavulanate without a concomitant decrease in cephalosporin susceptibility.

Transparency declarations

None to declare.

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